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CpG-induced immunomodulation and intracellular bacterial killing in a chicken macrophage cell line

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Abstract

The immunostimulatory properties of synthetic CpG oligodeoxynucleotides (ODNs) have been studied in various mammalian models including humans and mice. However, little was known about effects of CpG ODNs on immune responses of chickens, a common avian species with important economical value in the poultry industry. In the present study, two CpG ODNs, 2006 and 1826, which show immunomodulating properties for humans and mice were tested using a chicken macrophage cell line (HD11). ODN 2006, which has been reported to be an optimal stimulatory sequence for humans, showed strong immunomodulatory effects on HD11 cells, whereas ODN 1826, a CpG sequence with optimal immunostimulatory effects on mice, had weak influences on HD11 cells. ODN 2006 also induced strong IL-6 and nitric oxide secretion by HD11 cells in both dose- and time-dependent manners. Intracellular killing of *Salmonella enteritidis* (SE) was also increased in ODN 2006-activated HD11 cells. Furthermore, HD11 cells had reduced proliferation and underwent apoptosis, which is contradictory to the effects of ODN 2006 on human and murine cells. N^G-monomethyl L-arginine (L-NMMA), an iNOS inhibitor, inhibited apoptosis of HD11 cells induced by ODN 2006, suggesting that this effect was likely mediated through an iNOS-dependent pathway. These results indicate that the differences in the responses of chicken HD11 macrophage cells to CpG ODNs compared to those of mammalian macrophages are species-related, and the potential of CpG ODNs as immunomodulators in poultry needs to be further explored.

Keywords: Chicken; Macrophages; Phagocytosis; Apoptosis

1. Introduction

In 1992, Yamamoto et al. [38] first reported that bacterial DNA (bDNA) had immune stimulatory effects and could activate NK cells, while vertebrate DNA had no such effects. They attributed this phenomenon to the self-complementary palindromes contained in bDNA. Subsequently it was found that the immunostimulatory properties of bDNA are

Abbreviations: bDNA, bacterial DNA; BrdU, 5-bromo-2′-deoxyuridine; CFU, colony forming unit; GFP, green fluorescent protein; LAL, *Limulus* amebocyte lysate; L-NMMA, N^G-monomethyl L-arginine; ODN, oligodeoxynucleotide; PI, propidium iodide; SE, *Salmonella enteritidis*; TLR, toll-like receptor.

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actually due to the highly enriched unmethylated CpG dinucleotide content, and are independent of secondary or tertiary structures [19]. Although occurring at extremely low frequencies, vertebrate DNA also contains CpG dinucleotides, which normally exists in a consensus pattern of CCGG with the 5′-C methylated. The immune stimulatory activity of unmethylated CpG in bDNA is particularly dependant upon base motifs, and generally exists in the form of XCGY, in which X and Y can be any base except C and G, respectively [19]. Increasing the number of such CpG motifs in a sequence leads to enhanced stimulatory activity. Abolishing or methylating CpG motifs results in complete loss of immune stimulation. In addition, different animal species respond to different CpG motifs. Optimal CpG motifs for rodent immune cells normally are flanked by two purines at the 5′ end and two pyrimidines at the 3′ end, whereas the best CpG motif for human cells is GTCGTT [16,19,20].

Synthetic unmethylated CpG oligodeoxynucleotide (ODNs) sequences can mimic the immunostimulatory activity of bDNA, and modulate immune responses of a wide variety of cells including lymphocytes, macrophages/monocytes, dendritic and natural killer cells. The immunomodulatory effects of CpG ODNs include increasing proinflammatory cytokine production, up-regulating cell surface antigen expression, and promoting cell proliferation in many mammalian models [20,31,33]. Both in vitro and in vivo studies also demonstrate that CpG ODNs can directly elicit polyreactive IgM production, enhance responses of effector cells of innate immunity, and induce Th1-biased cytokines such as IFN-γ and IL-12, which lead to protective innate and adaptive immune reactions against various bacterial and parasite pathogens such as *Listeria*, *Francisella*, and *Leishmania* [9,17,18,35,40]. Moreover, CpG ODNs augment Ag-specific T and B cell responses, increase Ag-specific Ab titers and $CD8^+$ CTL responses, and synergize Ag-driven Th1 immunity, suggesting that CpG has great potential as a powerful vaccine adjuvant [3,7,18,23,24,40].

Salmonellosis is a major public health problem that causes severe food-borne diseases and tremendous economic loss every year. *Salmonella enteritidis* (SE) contaminated eggs are a source of *Salmonella* infection for humans. SE can persist in laying hens for a long time without causing serious illness. The mechanisms by which SE evades the host immune system are unclear. Current commercial SE vaccines using killed SE organisms are not effective and do not completely prevent SE infection, colonization and shedding in chickens. New strategies to improve the efficiency of traditional vaccines as well as develop novel vaccines against SE infection in chickens are needed. The potential of CpG ODNs as effective immunostimulatory agents and vaccine adjuvants has been suggested in mammalian systems. However, very limited information is available on the potential of CpG ODNs for the improvement of poultry health and food safety. In this study, the in vitro effects of CpG ODNs were investigated in chicken macrophages using a virus transformed cell line, HD11. The results suggest that chicken macrophages demonstrate similar and yet distinct responses to CpG ODNs compared to mammalian cells.

2. Materials and methods

2.1. ODNs

The CpG-containing ODNs used in this study were selected based on published reports (Table 1), and were commercially synthesized (Invitrogen Life Technologies, Gaithersburg, MD). For each CpG ODN, its control sequence has the same base composition, but with the CpG motif disrupted. All synthetic ODNs used in this study were tested for endotoxin contamination upon reconstitution using a *Limulus amebocyte lysate* (LAL) assay kit (Sigma Chemical Co., St Louis, MO), and all contained less than 0.15 EU/mg endotoxin.

2.2. Cells and cell culture

The HD11 chicken macrophage cell line and murine B9 hybridoma cells were both maintained in RPMI-1640 medium with 10% FCS (v/v), nonessential amino acids (1X), sodium pyruvate (1 mM), L-glutamine (2 mM), antibiotic/antimycotic (1X), and gentamicin (0.05 mg/ml). Because B9 is an IL-6-dependent cell line, recombinant human IL-6 (Roche Molecular Biochemicals, Indianapolis, IN)

Table 1
Summary of selected CpG sequences and their modulatory effects on HD11 cells

ODN code	Reference	Sequence (5′ → 3′)	Length (in bases)	Induced responses[a]	
				IL-6 secretion	NO_2 production
2006	25,28	TCGTCGTTTTGTCGTTTTGTCGTT	24	++++	++++
Control sequence I	–	GTCTGTCTTTGGTCTTTTGGTCTT	24	–	–
1826	25,28	TCCATGACGTTCCTGACGTT	20	+	+
Control sequence II	–	TCCATGGACTTCCTGGACTT	20	–	–

[a] Indicates various magnitudes of relative responses.

was always added at a concentration of 10 U/ml (1 U = 10 ρg) for routine maintenance. During CpG stimulation, HD11 cells were suspended in RPMI-1640 medium with 2% FCS at 2×10^5 cells/well in the presence of 0.1–6 μM CpG ODNs. In the blocking experiments, a final concentration of 500 μM N^G-monomethyl L-arginine (L-NMMA) (Sigma) was added to the cell culture in the presence/absence of ODNs at the beginning. Each treatment was conducted in triplicate.

2.3. Measurement of IL-6 and nitric oxide

IL-6 released by CpG-stimulated HD11 cells was measured using IL-6-dependent B9 hybridoma proliferation [36]. Briefly, after washing to remove existing IL-6, B9 cells were suspended in IL-6-free RPMI-1640 medium with 10% FCS, and plated at 10^4 cells/100 μl/well with 25 μl/well recombinant human IL-6 or HD11 cell conditioned media at 37 °C, 5% CO_2 for 72 h. Cell proliferation was determined using the reduction of MTT and OD was measured at 570 nm. Nitric oxide production by activated HD11 cells was assessed as nitrite content in conditioned media using Griess reagent as described [8]. Sodium nitrite was used as the standard.

2.4. Cell surface antigen expression

At 24 h post-stimulation by ODNs, HD11 cells were washed, aliquoted, and stained for 30 min on ice with each of five mAbs as follows: anti-chicken K55 [5], anti-chicken K1 [6], anti-chicken MHC class I [6], and anti-chicken MHC class II [22]. K55 recognizes a cell surface antigen that is expressed on chicken leukocytes while K1 recognizes a cell surface antigen on chicken macrophages (function unknown). Mouse anti-human Ig was used as isotype control. Binding of primary antibodies was followed by staining with FITC-conjugated anti-mouse IgG as secondary Ab for another 30 min on ice. The cells were then suspended with paraformaldehyde (4%) in PBS and analyzed by an Epics Elite flow cytometer (Beckman Coulter) with 10,000 cells analyzed per sample.

2.5. Phagocytosis and bacterial killing assays

Phagocytic and intracellular killing activities of HD11 cells were measured according to a previously described method with some minor modifications [29]. SE strain 338, a phage type 4 associated with human infection, was labeled with a red shifted derivative of the *gfp* gene under control of a *lac* promoter. A plasmid encoding kanamycin resistance and containing the above construct (pVLacGreen) was transferred to strain 338 by conjugation. Green fluorescent protein (GFP) engaged SE (GFP-SE) was grown in trypticase soy broth with yeast extract containing 50 μg/ml kanamycin (Difco) at 37 °C overnight, and standardized to an optical density of 0.7 at 550 nm. Following 24 h incubation with ODNs, HD11 cells were washed and resuspended into antibiotic free RPMI-1640 medium with 10% FCS. The cells were then mixed with GFP-SE suspension at the ratio of HD11 cells:GFP-SE, 1:100 in RPMI-1640 containing 10% FCS and no antibiotics, and incubated on a rotator at 37 °C, 5% CO_2 for 2 h. After washing thoroughly, the cells were reconstituted in gentamicin (10 μg/ml) containing medium and incubated again for up to 48 h. Aliquots of cells were stained by

propidium iodide (PI), and then analyzed by two-color flow cytometry (Beckman Coulter) with 10,000 cells analyzed at each time. Viable cells infected with GFP-SE were identified by fluorescence. Using an auto-clone device with the flow cytometer, 200 cells/treatment/well were sorted into three replicate wells in a 96-well tissue culture plate containing 100 μl/well Triton X-100 (0.01%). Sorted HD11 cells were then lysed for 20 min at room temperature, to release intracellular GFP-SE which were plated on trypticase soy agar overnight to determine colony forming units (CFU) of bacteria inside HD11 cells. The total CFU counts from all triplicates were averaged for each treatment and each time point. The average CFU values from 2 h phagocytosis served as the baseline for intracellular bacterial recovery (0 h intracellular bacterial recovery).

2.6. Proliferation assay

Effects of ODNs on the growth of HD11 cells were determined using a cell proliferation assay kit (Amersham) according to manufacturer's instruction. After culture with ODNs for 22 h, HD11 cells were incubated with the labeling reagent containing 5-bromo-2′-deoxyuridine (BrdU) and 5-fluoro-2′-deoxyuridine for an additional 2 h. Following fixing and blocking, the cells were further labeled by monoclonal anti-BrdU with nuclease at room temperature for 1 h. The BrdU incorporation was determined by detection of bound Ab using peroxidase conjugated anti-mouse IgG_{2a} and ABTS, which yielded a soluble greenish color at 405 nm.

2.7. Apoptosis assay

Apoptosis was determined using a commercial Annexin V-FITC apoptosis detection kit (BD Biosciences). HD11 cells that underwent an apoptotic death were identified based upon the high affinity of Annexin V for the membrane phospholipid phosphatidylserine, and were analyzed by double positive staining with Annexin V-FITC and PI using flow cytometry.

2.8. Caspase-9 activity assay

After 18 h co-incubation with CpG or control ODNs, the caspase-9 activity in activated cells was assessed using a caspase-9 colorimetric assay kit according to the manufacturer's instruction (R&D System). All assays were performed in triplicate.

2.9. Statistical analysis

Statistical significance was evaluated using Student's *t*-test (P values ≤ 0.05). All the studies were conducted in triplicate, and data expressed as the mean ± standard error.

3. Results

3.1. Effects of CpG ODNs on activation of HD11 cells

ODN 2006 and ODN 1816 are reported as optimal stimulatory motifs for humans and mice, respectively. Screened by both nitrite and IL-6 assays, ODN 1826 induced minimal NO_2 and IL-6 production in HD11 cells (Table 1). In contrast, ODN 2006 stimulated very strong production of NO_2 and IL-6 in both dose- and time-dependent manners (Fig. 1A–D). None of the control sequences used in this study had any effects on these two parameters (Table 1). Based on these results, we focused on ODN 2006 and further characterized its immunomodulatory effects on chicken HD11 cells.

3.2. Effects of ODN 2006 on HD11 cell surface antigen expression

K55 recognizes a cell surface antigen that is expressed on chicken leukocytes while K1 recognizes a chicken macrophage marker. Although the expression of the antigens recognized by these antibodies can be up-regulated by IFN-γ, their exact identity, and therefore, function are unclear. In Fig. 2, the expression of the K55-recognized marker on CpG-stimulated HD11 cells showed a dose-dependent increase in the presence of ODN 2006 (1–3 μM), which declined to the basal level at a higher concentration (6 μM). The expression of K1 on ODN 2006-stimulated HD11 cells also showed a tendency of increase (not significantly). However, ODN 2006 had no apparent effects on both MHC class I and II expression on activated HD11 cells.

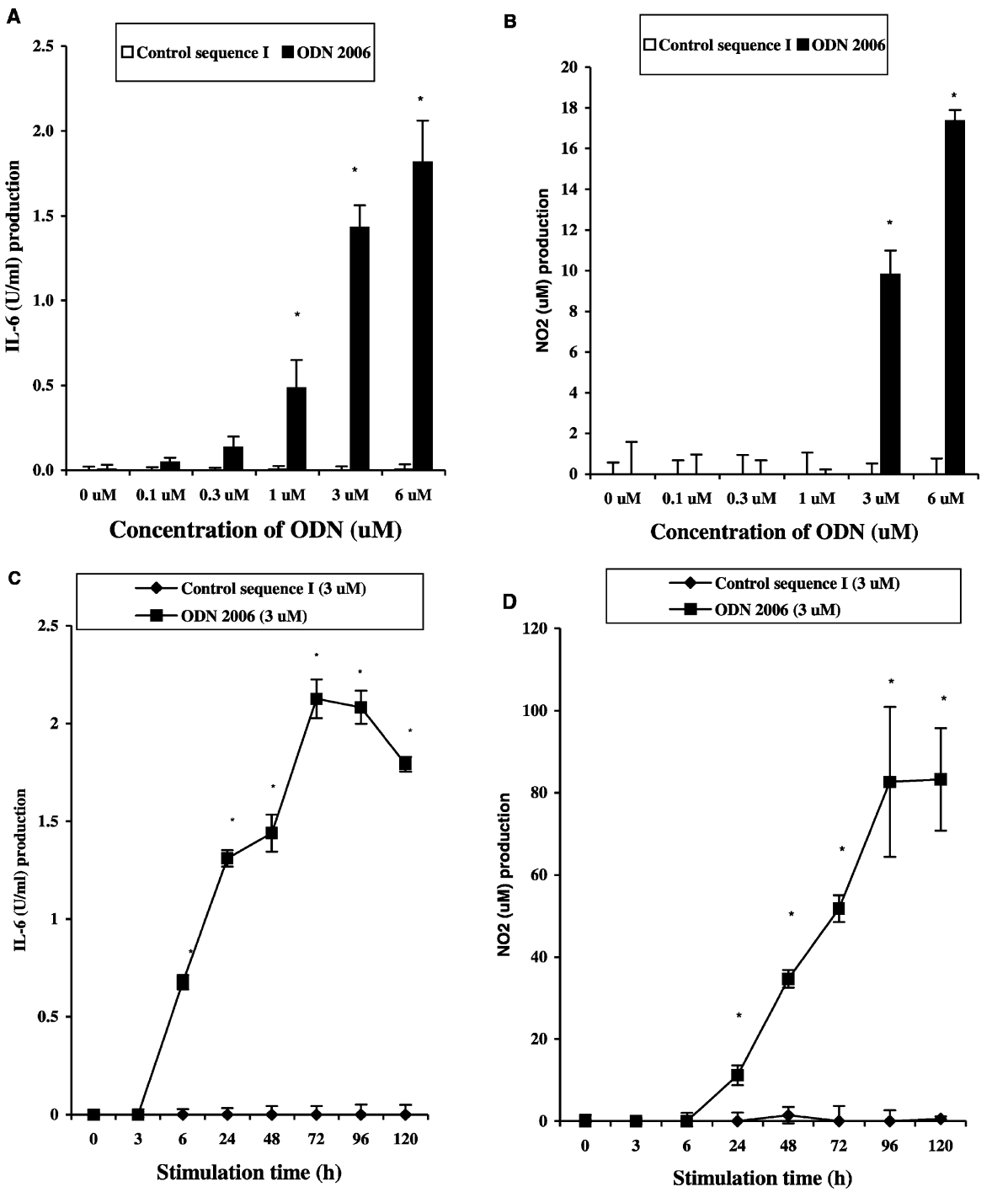

Fig. 1. Responses of HD11 cells to CpG motifs. HD11 cells were subjected to various concentrations of CpG ODN 2006 or control sequence I for 24 h and conditioned media were collected for IL-6 (A) and NO_2 analysis (B). The time courses of IL-6 (C) and NO_2 (D) production by HD11 cells stimulated with 3 μM CpG ODN (2006) or control sequence I were also determined. Data were expressed as mean ± standard error ($n = 3$), and were analyzed by Student's *t*-test. Significance (*) was considered as $P \leq 0.05$ when compared to the paired treatment of control sequence I.

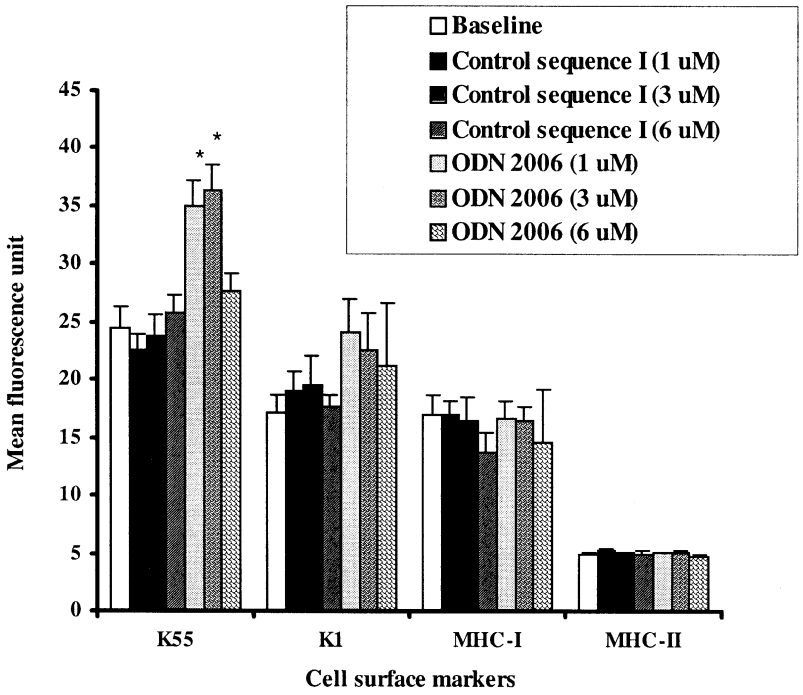

Fig. 2. Cell surface antigen expression on HD11 cells stimulated by synthetic CpG ODNs. HD11 cells were treated with various concentrations of CpG ODN 2006 or control sequence I for 24 h, and then stained with Abs against different cell surface markers for flow cytometry. Data were expressed as mean fluorescence units (log scale) ± standard error ($n = 3$), and were analyzed by Student's t-test. Significance (*) was considered as $P \leq 0.05$ when compared to the baseline.

3.3. Phagocytosis and intracellular killing of GFP-SE by HD11 cells

The effects of CpG activation on phagocytosis and bactericidal activities of HD11 cells were evaluated using GFP-expressing SE. ODN 2006 had no obvious influence on the phagocytic capability of HD11 cells toward GFP-SE. Although all treatments showed decreased intracellular bacteria recoveries over time, ODN 2006-stimulated HD11 cells had significantly lower counts of intracellular GFP-SE recovered at longer incubation times (Fig. 3), indicating that ODN 2006 stimulation may aid in intracellular killing of SE by HD11 cells in the long term.

3.4. Effects of 2006 on proliferation and apoptosis of HD11 cells

ODN 2006 treatment arrested HD11 cell growth in a dose-dependent fashion (Fig. 4A), which was different than previous observations in mammalian systems that CpG could inhibit apoptosis and promote cell proliferation. ODN 2006 treated cells had distinctive morphological changes compared to baseline and control sequence incubated cells, including cell shrinking and membrane blebbing (data not shown). FACS analysis showed that there was a large population of cells that stained double positive for both Annexin V-FITC and PI in ODN 2006 treated HD11 cells at 24 h post-stimulation, which had a dose-dependent increase at concentrations between 1–6 μM of ODN 2006 (data not shown and Fig. 4B and C). Likewise, the activity of caspase-9, a cysteine protease involved in programmed cell death, was also significantly increased in ODN 2006-activated HD11 cells (Fig. 5B). No significant differences were observed in the basal levels of cell death between medium-treated baseline and control sequence-treated samples (Fig. 4B).

To determine the possible role of NO_2 production in CpG induced apoptosis in chicken HD11 cells, L-NMMA, an inhibitor of iNOS was introduced into

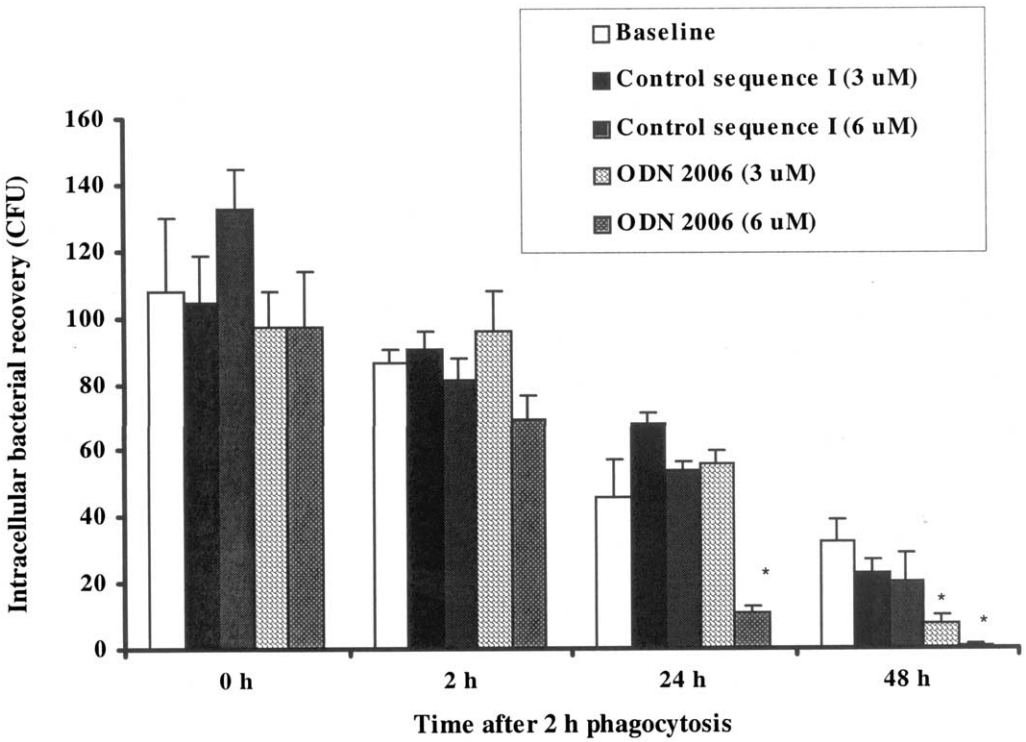

Fig. 3. Effects of CpG motifs on intracellular recovery of SE from infected HD11 cells. HD 11 cells were stimulated with various concentrations of CpG ODN 2006 or control sequence I for 24 h, and then infected with GFP-SE for 2 h. Extracellular SE was killed by gentamicin (10 μg/ml). GFP-SE infected HD11 cells sorted by flow cytometry were lysed using Triton X-100 (0.01%), and were plated on trypticase soy agar overnight to determine CFU of released intracellular SE from HD11 cells. The average CFU values from 2 h phagocytosis served as the baseline for intracellular bacterial recovery (0 h intracellular bacterial recovery). Data were expressed as mean ± standard error ($n = 3$), and were analyzed by Student's *t*-test. Significance (*) was considered as $P \leq 0.05$ when compared to the blank at the same time point. The results presented here are the representative of at least three individual experiments.

the cell culture. L-NMMA blocked the release of NO_2 by ODN 2006-activated HD11 cells (Fig. 5A), but had no influence on ODN 2006 stimulated IL-6 production (data not shown). Staining for annexin V also showed that L-NMMA inhibited ODN 2006-induced apoposis in HD11 cells (Fig. 5C), which was confirmed by the caspase-9 activity assay (Fig. 5B).

4. Discussion

This study is the first report to systemically investigate the immunostimulatory effects of CpG ODNs on chicken macrophages. The data indicate that the responses of chicken macrophages to CpG ODNs show both similarities and differences to mammals. ODN 2006, an optimal CpG sequence for humans, had strong stimulatory effects on HD11 cells as demonstrated by increased secretion of the proinflammatory cytokine IL-6, enhanced NO_2 release, upregulated cell surface marker expression, and increased intracellular bacterial killing. The optimum dose range of ODN 2006 in HD11 cells was between 0.1–6 μM, and the sensitivity of these in vitro reactions in chicken cells were similar to mammalian cells [12,25]. However, rodent optimized sequence ODN 1826 had weak influences on the functions of HD11 cells. Previous publications also reported that CpG ODNs that are favored by rodents work poorly in primates [1,11,12,33]. A number of laboratories

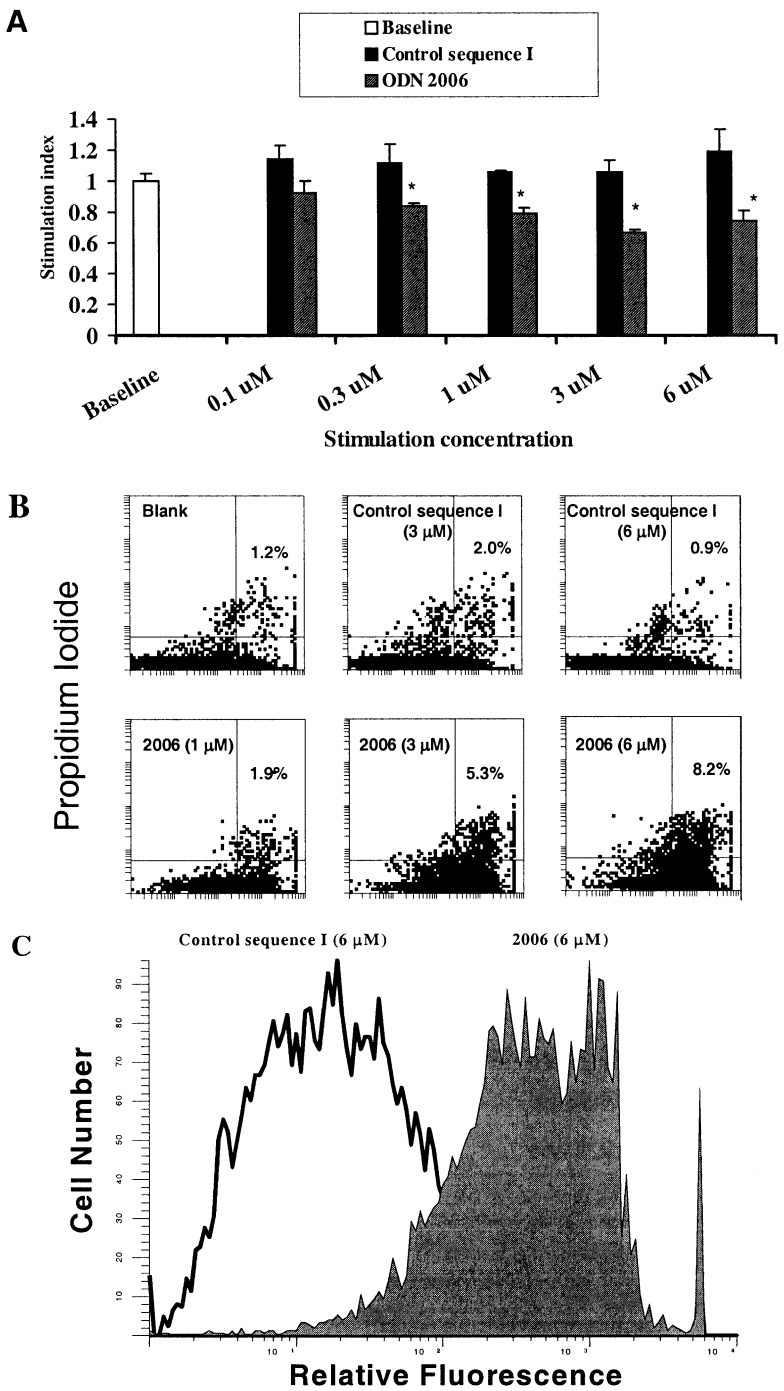

Fig. 4. Influence of CpG motifs on proliferation and apoptosis of activated HD11 cells. HD 11 cells were stimulated with various concentrations of CpG ODN 2006 or control sequence I for 24 h, and analyzed for proliferation using BrdU incorporation ELISA (A), or apoptosis using propidium iodide and Annexin V-FITC staining (B and C): open area = control sequence I (6 μM); shaded area = ODN 2006 (6 μM). Proliferation results were expressed as mean ± standard error ($n = 3$), and were analyzed by Student's t-test. Significance (*) was considered as $P \leq 0.05$ when compared to the baseline. The results presented here are representative of at least three individual experiments.

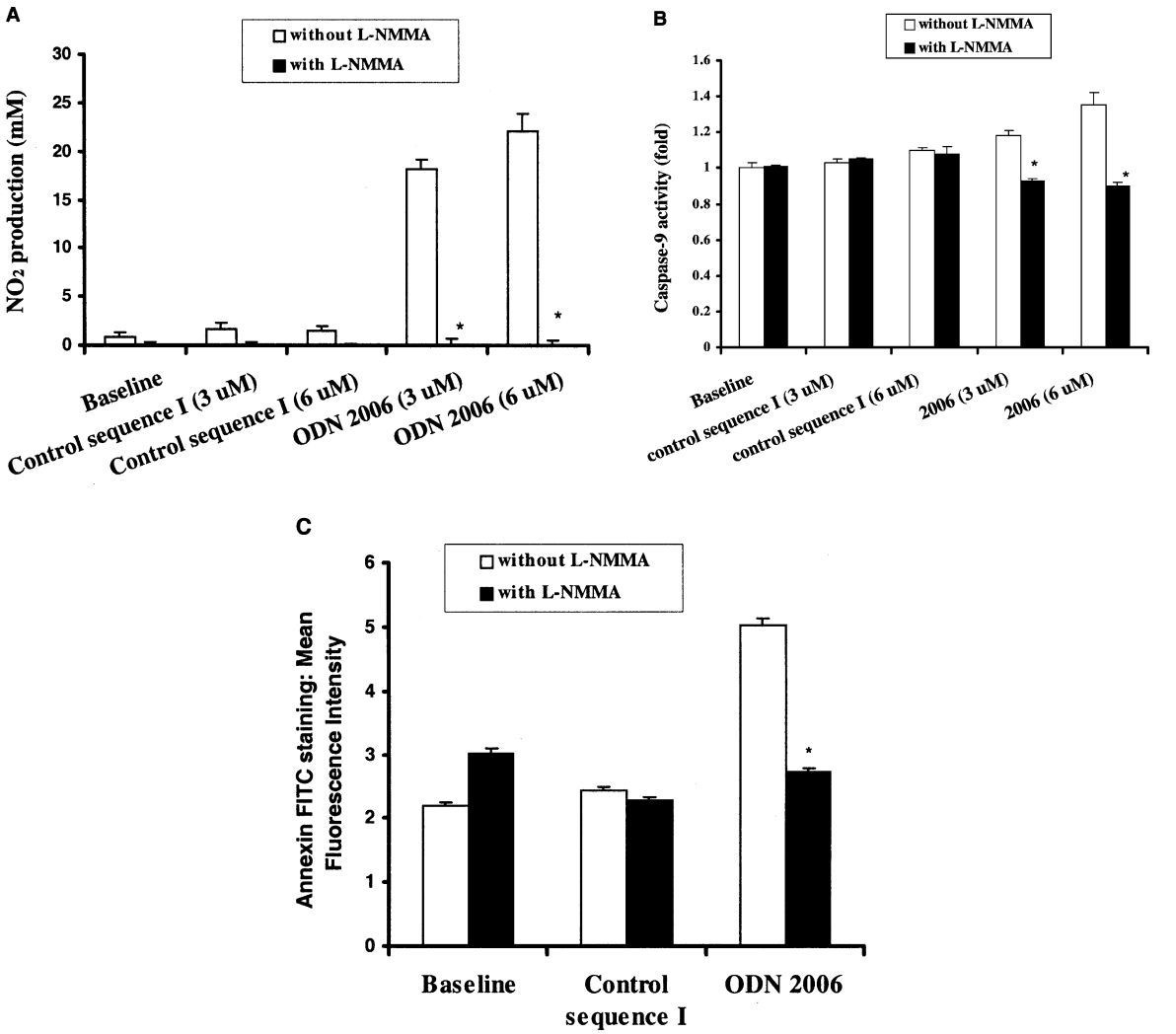

Fig. 5. Influences of L-NMMA on CpG induced nitrite production and cell death. After 24 h incubation with both ODNs and L-NMMA (0.5 mM), the HD11 conditioned media were collected for analysis of NO_2 production (A). The cells were than harvested for caspase-9 activity assay (B) or Annexin V-FITC and PI staining (C). Data were expressed as mean ± standard error ($n = 3$), and were analyzed by Student's t-test. Significance (*) was considered as $P \leq 0.05$ when compared to the treatment without L-NMMA at the same concentration. The results presented here are the representative of at least three individual experiments.

have reported that different animal species have their preferred CpG ODNs, and many of these CpG sequences have been identified including preferred motifs for human, rhesus monkey, mouse, rabbit, bovine, feline, swine, and fish [4,11,12,15,28,33]. In general, motifs containing at least one GTCGTT sequence show activities on immune cells of most vertebrate species except mice, where the preferred motif is GACGTT [28]. Consistent with the publications from other laboratories, we also observed that ODN 2006, a sequence with three GTCGTT repeats, showed much stronger immunomodulatory effects on chicken HD11 cells than ODN 1826, an optimal motif for murine with two GACGTT repeats. The selectiveness in response to different CpG motifs by different vertebrate species has been attributed to the evolutionary diversity in recognition of bDNA [11,12,28,33].

In the long history of evolution, nature has developed highly specialized host defensive mechanisms against infection and inflammation for each species. Toll-like receptors (TLRs) are a group of highly conserved transmembrane proteins in vertebrates, which recognize and respond to different microbial components. To date, 10 TLRs (TLR1-10) have been identified in mammals, each having its own specific target during microbial invasion. For instance, TLR3, TLR4 and TLR5 are the essential receptors in recognition of viral double-stranded RNA, bacterial LPS and flagellin, respectively [30,34]. TLR2 along with other TLRs such as TLR6 and TLR1 forms different functional heterodimers and recognizes a wide variety of pathogen-specific molecular patterns (PAMPs) including peptidoglycan and lipoproteins [30,34]. TLR7 might be implicated in viral infection [14], whereas TLR9 is the key receptor in CpG and bacterial DNA mediated activation of immune cells [13,30,34]. Although TLRs are highly conserved in most vertebrates, the variations in their amino acid sequences may lead to selective responsiveness to different microbial components as well as bacterial CpG DNAs [11,13,32]. Recently chicken TLR type 1 and 2 have been identified, which show about 45% homology to human TLR2 at the protein level [10]. However, it was also found that chicken TLR type 2 possesses activities of both human TLR2 and TLR4, which recognizes two major bacterial cell wall components, lipoproteins and LPS [10]. It appears that the evolutionary divergence not only leads to the selectivity of different species in recognition of specific CpG motifs, but also likely contributes to the differences in their susceptibility or resistance to a variety of pathogens.

Previous publications also suggested that bDNA and CpG ODNs are mitogenic for mammalian mononuclear cells, and block spontaneous apoptosis in both DC and B cells [19,20,26,33,39]. The mechanisms by which CpG ODNs inhibit apoptosis are not clearly understood, and both caspase-dependent and -independent regulations are believed to be involved in CpG-activated cell survival [26,27]. In the present study, we observed that ODN 2006 CpG actually arrested chicken HD11 cell growth and induced programmed cell death. Several possibilities might explain this difference seen between mammalian and avian systems. First, macrophages may respond to bDNA and CpG-ODNs differently from DC and B cells. Second, it may be that the evolutionary divergence in both recognition and interaction with bDNA and CpG motifs may contribute to this species-related difference. And third, a virus-transformed chicken macrophage cell line (HD11) was used in this study in contrast to primary mammalian leukocytes used by other laboratories. HD11 is an avian acute leukemia virus transformed cell line initially established and characterized by Beug et al. [2], which has been widely used to understand chicken immune mechanisms in vitro. Although HD11 cells retain many morphological and functional characteristics of primary chicken macrophages [2,37], like other spontaneously mutated or virally immortalized cells, their DNA expression may have changed during transformation. This in turn may result in turning on certain intracellular factors that might be involved in the signal transduction of CpG activated apoptosis in HD11 cells. Further investigations with primary cell types are required to confirm this hypothesis.

Apparently, ODN 2006-induced apoptosis in HD11 cells is regulated through an iNOS-dependent pathway since apoptosis could be inhibited by L-NMMA, an inhibitor of iNOS. Although oxygen and nitrogen intermediates released by phagocytic cells are part of the host defense against microbial invasion, uncontrolled production of these highly active small molecules can cause damage to the host itself [21]. Our results, obtained in this study, reflected the double-edged effects of NO_2 during infection. High levels of NO_2 production induced by ODN 2006 not only resulted in enhanced intracellular bacterial killing, but also led to increased cytotoxicity in HD11 cells.

SE has long been a serious problem in food safety, the mechanism by which it is able to persist in chickens and evade the immune system is still not completely understood. The data provided in this study suggest that CpG ODNs could drive SE-infected HD11 cells to undergo programmed cell death, leading to enhanced intracellular clearance of SE. This finding may provide a new strategy for developing therapeutics or more efficient vaccines against SE in poultry.

Acknowledgements

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